Published in final edited form as:
Mol Microbiol. 2008 June ; 68(6): 1535–1546. doi:10.1111/j.1365-2958.2008.06250.x.

Plasmodium falciparum Sec24 marks transitional ER that exports a model cargo via a diacidic motif

Marcus C. S. Lee[1], **Pedro A. Moura**[1], **Elizabeth A. Miller**[2], and **David A. Fidock**[1,3,*]

1*Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA*

3*Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA*

2*Department of Biological Sciences, Columbia University, New York, NY 10027, USA*

Summary

Exit from the endoplasmic reticulum (ER) often occurs at distinct sites of vesicle formation known as transitional ER (tER) that are enriched for COPII vesicle coat proteins. We have characterized the organization of ER export in the malaria parasite, *Plasmodium falciparum*, by examining the localization of two components of the COPII machinery, PfSec12 and PfSec24a. PfSec12 was found throughout the ER, whereas the COPII cargo adaptor, PfSec24a, was concentrated at distinct foci that likely correspond to tER sites. These foci were closely apposed to *cis*-Golgi sites marked by PfGRASP-GFP, and upon treatment with brefeldin A they accumulated a model cargo protein via a process dependent on the presence of an intact diacidic export motif. Our data suggest that the cargo-binding function of PfSec24a is conserved and that accumulation of cargo in discrete tER sites depends upon positive sorting signals. Furthermore, the number and position of tER sites with respect to the *cis*-Golgi suggests a co-ordinated biogenesis of these domains.

Introduction

Worldwide, malaria afflicts an estimated 500 million people each year, resulting in over one million deaths (Snow *et al*., 2005). Clinical manifestations of the disease are caused by asexual blood stages of the intracellular parasite *Plasmodium falciparum* that replicate in host erythrocytes. Upon invasion of the host cell, the parasite becomes enclosed in a parasitophorous vacuole, which houses the parasite as it develops to form between eight and 24 daughter cells over the course of ~48 h (Miller *et al*., 2002). This intraerythrocytic development is accompanied by distinct morphological changes to both the parasite and the host erythrocyte. As the parasite matures from the early cup-like `ring-stage' into the trophozoite stage, it becomes more spherical and acquires a large acidic digestive vacuole that is the site of hemoglobin degradation a process that is required to sustain parasite growth. Later during development, schizogony is characterized by multiple rounds of nuclear division and the biogenesis of specialized apical secretory organelles that promote invasion of daughter merozoites into a new host cell (Bannister *et al*., 2000a). In addition to these various internal membrane remodelling events, the parasite manipulates the surface of the host erythrocyte by

*For correspondence. E-mail E-mail: df2260@ columbia.edu; Tel. (+1) 212 305 0816, Fax (+1) 212 305 4038..

Supplementary material This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2958.2008.06250.x
Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

deploying secreted proteins that function in cytoadherence to endothelial receptors. This enables infected erythrocytes to sequester in the microvasculature and avoid splenic clearance (Marti *et al*., 2005; Tonkin *et al*., 2006). Despite the importance of protein secretion and membrane remodelling to malaria pathogenesis, the organization of the parasite endomembrane system and the molecular mechanisms that direct protein trafficking to distinct destinations remain poorly understood.

In eukaryotic cells, proteins destined for the extracellular environment, or for the various membranes of the secretory pathway, are translocated into the endoplasmic reticulum (ER) prior to being selectively captured into transport vesicles for delivery to downstream compartments. Vesicle budding from the ER membrane is mediated by cytoplasmic coat proteins, termed the COPII coat, which both deform the membrane and select secretory proteins for incorporation into a nascent vesicle. The COPII coat comprises five proteins: Sar1 is a GTPase that regulates coat recruitment; the Sec23/Sec24 heterodimer modulates Sar1 activity and promotes cargo capture; and Sec13/Sec31 forms the outer scaffold that likely drives the formation of a spherical vesicle (Bonifacino and Glick, 2004; Gurkan *et al*., 2006; Lee and Miller, 2007). Capture of cargo proteins into COPII vesicles is often mediated by sorting signals that interact directly with Sec24. At least three independent cargo binding sites on Sec24 interact with a variety of ER export motifs found on different membrane proteins (Miller *et al*., 2003; Mossessova *et al*., 2003; Mancias and Goldberg, 2007). As an alternative to signal-mediated packaging, secretory proteins may also leave the ER by a less efficient, passive mechanism known as bulk flow (Martinez-Menarguez *et al*., 1999). This type of ER exit likely results from random sampling of the ER membrane and lumenal contents during vesicle formation, but its contribution to general secretion remains poorly defined.

Several components of the COPII coat have been identified in *P. falciparum*: PfSar1, PfSec23 and the `outer' coat components PfSec31 and PfSec13 (Albano *et al*., 1999; Adisa *et al*., 2001; 2007; Wickert *et al*., 2003; Struck *et al*., 2008). Although PfSar1, PfSec23 and PfSec31 were initially localized both within the parasite as well as to parasite-derived structures termed Maurer's clefts in the host cell cytosol, more recent evidence using GFP fusions of PfSar1 suggests an exclusive localization to the parasite ER (Adisa *et al*., 2007). The observations of host-localized COPII proteins may have resulted from antibody cross-reactivity to Maurer's cleft constituents (Adisa *et al*., 2007). PfSar1-GFP appears to be distributed throughout the ER, whereas PfSec13-GFP was localized to distinct regions that may correspond to sites of vesicle budding (Adisa *et al*., 2007; Struck *et al*., 2008).

In many eukaryotes, the ER is organized into distinct zones of vesicle formation known as transitional ER (tER). These tER sites are devoid of ribosomes and are enriched for the COPII machinery (Palade, 1975; Orci *et al*., 1991). Although the basic mechanism of COPII vesicle formation is likely to be universal, the higher-order organization of tER sites and their relationship with the Golgi differs dramatically across species. *Saccharomyces cerevisiae* appears to lack a distinct tER and budding occurs promiscuously throughout the ER, whereas *Pichia pastoris*, another yeast, possesses few discrete sites that are enriched in COPII proteins (Rossanese *et al*., 1999; Bevis *et al*., 2002).

To better define the nature of ER export in malaria parasites, we have examined two proteins involved in COPII vesicle formation, namely the Sar1 GTP exchange factor, PfSec12, and the COPII cargo adaptor, PfSec24a, with respect to their subcellular localization. Sequence conservation of a key cargo binding site on PfSec24a led us to address whether ER export in *P. falciparum* utilizes a signal-mediated mechanism similar to that in yeast and mammalian cells. Our findings suggest that ER export in this malaria parasite is highly organized, and provide insight into the molecular basis of membrane and protein secretion in this important intracellular pathogen.

Results

PfSec12: the guanine nucleotide exchange factor for PfSar1

In studying the organization of the early secretory pathway in *P. falciparum*, we first identified the parasite orthologue of the ER membrane protein Sec12, an upstream effector in COPII vesicle biogenesis (Barlowe and Schekman, 1993). Homology searches of the *P. falciparum* genome identified a single gene, PF11_0171, that encodes a protein sharing ~15% sequence identity to both yeast and mammalian Sec12, with several conserved features. These include a tetra-glycine motif and predicted WD40 repeats in the Sar1-binding cytosolic domain, and a C-terminal transmembrane segment (Chardin and Callebaut, 2002). PfSec12 has a predicted mass of 56 kDa and is encoded by a single exon of 1458 bp, with orthologues present in the genomes of other *Plasmodium* species.

We generated a transgenic parasite line expressing PfSec12 with an N-terminal fusion to monomeric DsRed (mRFP), and tested the ability of recombinant parasite Sar1 to capture mRFP-PfSec12 from a detergent extract. Parasites were isolated from host erythrocytes by saponin lysis, which at low concentrations selectively permeabilizes the host plasma membrane and the parasitophorous vacuole membrane, leaving the parasite intact. The washed parasite pellet was then extracted with either SDS or the non-ionic detergent octylglucoside, which is permissive for the detection of protein-protein interactions. Immunoblot analysis of the SDS-solubilized membranes with anti-DsRed antisera detected a single protein of the expected molecular mass in transfected parasite extracts but not in the untransfected control (Fig. 1A). Octylglucoside-solubilized proteins were incubated with either GST-PfSar1 or GST alone immobilized on glutathione-agarose beads, and interacting mRFP-PfSec12 was detected by immunoblot. GST-PfSar1 but not GST alone was able to efficiently isolate PfSec12 (Fig. 1B). Despite the low sequence identity between parasite and yeast Sec12, a robust interaction between mRFP-PfSec12 was also observed with beads coated with yeast Sar1 (Fig. 1C), suggesting conservation of the Sar1-Sec12 interface.

PfSec12 localizes to the general ER

To localize PfSec12, we developed a transgenic line expressing GFP-PfSec12 from the endogenous *pfsec12* 5′ untranslated region (UTR) and examined expression by live cell imaging. Over the course of the ~48 h intraerythrocytic cycle, GFP-PfSec12 was distributed in a pattern characteristic of the parasite ER (van Dooren *et al*., 2005), with a perinuclear distribution in ring stage parasites and additional elongated extensions in mature trophozoite stages. During schizogony, the ER forms a mesh-like network, and GFP-PfSec12 was observed encircling each individual nucleus (Fig. 1D).

As PfSec12 did not appear to be distributed uniformly throughout the ER in all parasites, we compared the pattern of PfSec12 with that of GFP-SDEL, which localizes throughout the general ER. GFP-SDEL is targeted to the ER by virtue of a signal peptide that directs translocation into the ER lumen and a C-terminal SDEL motif that mediates retrieval from the Golgi, resulting in colocalization with the ER chaperone BiP (van Dooren *et al*., 2005). Parasites were co-transfected with plasmids for mRFP- PfSec12 and GFP-SDEL, which conferred resistance to Blasticidin S and WR99210 respectively. The distribution of PfSec12 coincided with that of GFP-SDEL, indicating that PfSec12 behaves as a general ER protein (Fig. 2A). Consistent with this distribution, GFP-PfSec12 similarly colocalized with BiP by immunofluorescence analysis (Fig. S1).

The *P. falciparum cis*-Golgi is marked by the peripheral membrane protein PfGRASP, which lies in close proximity to the ER marker BiP (Struck *et al*., 2005). We examined the localization of PfSec12 in relation to the Golgi by generating transgenic lines coexpressing mRFP-PfSec12

and PfGRASP-GFP. In live parasites we observed close apposition of PfGRASP-GFP with PfSec12 fluorescence, in particular within regions of the perinuclear ER (Fig. 2B). In several organisms, the Golgi is juxtaposed to sites of vesicle budding on the ER. To determine whether Golgi localization correlates with tER sites in *P. falciparum*, we next examined a component of the COPII coat.

The COPII component PfSec24 marks transitional ER sites

Sec24 is the cargo-binding adaptor of the COPII coat and forms a heterodimer with Sec23, the GTPase activating protein for Sar1. Multiple paralogs of Sec24 are present in the genomes of most organisms, likely expanding the repertoire of cargo proteins recognized by the COPII coat. We identified two putative Sec24 orthologues in the *P. falciparum* genome. PF13_0324 (PfSec24a) shares significant homology with the mammalian Sec24A/B iso-forms and yeast Sec24, whereas PFD0250c (PfSec24b) is less conserved overall and appears more closely related to the mammalian Sec24C/D family and the yeast Sec24 homologue Lst1 (Fig. S2). Both PfSec24a and PfSec24b share conserved residues within the core `trunk' domain involved in the Sec23-Sec24 interface, including a loop region containing the FLP residues that are highly conserved across a variety of species (Fig. S2). PfSec24b is predicted to be considerably larger than both PfSec24a and yeast Sec24, owing in part to an extended N-terminal domain, a region of unknown function that is highly variable among Sec24 proteins (Bi *et al*., 2002). Because of the large size of PfSec24b, and the generally high AT-content of the *P. falciparum* genome, we were unable to obtain an expression plasmid with PfSec24b that could be replicated stably in *Escherichia coli*, restricting our current analysis to PfSec24a.

PfSec24a was tagged by C-terminal fusion with either GFP or mRFP, based on an earlier study showing that yeast Sec24p retains function when similarly modified at the C-terminus (Huh *et al*., 2003). Because of difficulties in amplifying the endogenous 5′ UTR, expression of the PfSec24a fusion was driven by the 5′ UTR of the parasite syntaxin 17 homologue, which displays a similar transcriptional profile to *pfsec24a* and is predicted to have approximately three- to fourfold less transcript based on the average median intensity of the respective microarray probes (Bozdech *et al*., 2003; Ayong *et al*., 2007). Unlike PfSec12, which was distributed throughout the ER, PfSec24a marked distinct foci close to the nucleus that likely represent tER sites of vesicle budding (Fig. 3A). In parasites coexpressing GFP-PfSec12 and PfSec24a-mRFP, PfSec24a was closely associated with the peri-nuclear pool of PfSec12 (Fig. 3B).

The number of tER foci per parasite was quantified in ring, trophozoite and early schizont stages (Fig. S3A and B). Ring stage parasites (< 16 h postinvasion) typically contained 1-2 PfSec24a punctae, with mid-stage trophozoites showing 2-3 foci per cell. In late trophozoites, 3-4 PfSec24a foci were associated with a single nucleus, suggesting that multiplication of tER sites preceded nuclear division (Fig. S3A). The number of tER sites further increased at the onset of schizogony, with 6-8 tER sites typically observed in early schizonts containing two nuclei (Fig. S3B). As schizogony progressed, difficulties in reliably quantifying the number of nuclei precluded an accurate correlation between the number of tER and nuclei. At later stages of schizogony, however, each developing daughter merozoite ultimately inherited a single tER site (Fig. 3A, fourth and fifth rows).

Previous studies of other components of the *P. falciparum* COPII coat, including PfSar1, reported immunofluorescence labelling of structures within the host erythrocyte. However, a GFP fusion of PfSar1 was subsequently found only within the parasite ER (Albano *et al*., 1999; Adisa *et al*., 2001; 2007; Wickert *et al*., 2003). We were unable to detect any PfSec24a-GFP within the host erythrocyte, consistent with the exclusively intraparasitic localization of the `outer coat' component, PfSec13, fused to GFP (Struck *et al*., 2008).

Golgi are located in close apposition to PfSec24-marked tER sites

In organisms lacking long-range cytoskeleton-dependent movement between the ER and Golgi, tER sites are closely associated with the *cis*-Golgi, which receives new material by continuous delivery of COPII vesicles (Kirk and Ward, 2007). We examined the relationship between the tER and *cis*-Golgi in *P. falciparum* by generating a transgenic line coexpressing PfSec24a-mRFP and PfGRASP-GFP. Almost all cells showed equivalent numbers of PfSec24a and PfGRASP foci, which were always located in very close proximity, indicating that the Golgi develops adjacent to sites of vesicle budding from the ER (Fig. 4). Occasionally, two small PfSec24a foci could be seen flanking a single spot of PfGRASP-GFP, suggesting that fission of an existing tER site or *de novo* creation of a new tER site precedes Golgi duplication (Fig. 4, bottom row).

Potential conservation of a cargo binding site on PfSec24

Having defined tER sites through the localization of PfSec24a, we proceeded to test the conservation of the cargo-binding function of this COPII component. Structural and genetic data on the known cargo binding sites on yeast and mammalian Sec24 led us to inspect the sequences of PfSec24a and PfSec24b for conserved features, summarized in Fig. S4. Of the three known sites, the B-site appears to fulfil the main cargo-binding duties on Sec24. This region is highly conserved across species and mutagenesis of residues within this site compromises the export of multiple cargo proteins. The site is a basic pocket formed by a zinc-finger domain that engages in electrostatic interactions with acidic residues of ER export signals, including the diacidic DxE of the yeast Golgi protein Sys1, and the LxxLE-related signals of some ER-Golgi SNAREs (Miller *et al*., 2003; Mossessova *et al*., 2003). PfSec24a, but not PfSec24b, retains the four key arginine residues that form crystallographic contacts with acidic signals (Fig. S4). Given the high degree of conservation of the B-site of PfSec24a, we attempted yeast complementation experiments to determine whether PfSec24a could compensate for growth defects caused by either the complete loss of yeast Sec24 or the abrogation of specific cargo-binding functions. PfSec24a was unable to support viability in the absence of endogenous Sec24, likely owing to the poor expression levels obtained (data not shown). The lack of functional interspecies complementation may also stem from an inability to form productive heteromeric COPII complexes. Indeed, another parasite COPII protein, PfSar1, was also unable to complement the endogenous gene (Adisa *et al*., 2007).

We next asked whether the conserved B-site on PfSec24a functions in export from the ER. Because of the difficulty in reliably identifying export signals of secreted proteins, we examined the behaviour of a model protein, yeast Sys1, with a canonical diacidic signal. Sys1 recruits Arf-like GTPases to the Golgi and is a polytopic membrane protein with a cytosolic C-terminal domain that contains a DxE motif required for efficient ER egress (Votsmeier and Gallwitz, 2001; Behnia *et al*., 2004). We established transgenic lines expressing GFP fusions of Sys1 containing either an intact export motif (GFP-Sys1DxE) or one that had been mutated to alanine (GFP-Sys1AxA), and examined the localization of the reporter by live cell imaging to determine if the diacidic motif conferred efficient export from the ER. To delineate the ER, parasites were co-transfected with mRFP-PfSec12. Wildtype GFP-Sys1DxE was successfully exported from the ER. Unlike in yeast, the protein was transported beyond the Golgi, accumulating at the parasite plasma membrane (Fig. 5). Retention of Sys1 in the Golgi in yeast may be a function of the length of the transmembrane domains in relation to the thickness of the membrane bilayer (Bretscher and Munro, 1993), and differences in membrane lipid composition between yeast and *P. falciparum* may result in transit of Sys1 beyond the parasite Golgi.

GFP-Sys1AxA also accumulated at the plasma membrane, but a proportion of GFP-Sys1AxA was detected in the ER, suggesting a slower export of mutant Sys1 (Fig. 5). One explanation

for the similar localizations of the wild-type and mutant proteins is that both exit the ER, albeit at different rates that are poorly resolved by steadystate observations. In the absence of retention or retrieval mechanisms, proteins can leave the ER by a non-specific `bulk flow' mechanism. In support of this, GFP that is translocated into the parasite ER but that lacks a C-terminal retrieval motif ultimately accumulates in the parasitophorous vacuole (Adisa *et al*., 2003).

Brefeldin A causes retention of GFP-Sys1 at tER sites in a manner dependent on the ER export motif

The fungal metabolite brefeldin A (BFA) perturbs secretory traffic by preventing activation of the Arf1 GTPase, and treatment of *P. falciparum* parasites with BFA results in retention of secreted proteins within the ER (Crary and Haldar, 1992; Benting *et al*., 1994). Microscopic examination of BFA-treated lines expressing GFP-Sys1 revealed a striking difference in the distribution of the wild-type and mutant proteins: wild-type Sys1 was concentrated in 1-2 distinct foci, whereas the diacidic mutant was found in the perinuclear ER (Fig. 6A).

Transitional ER sites in mammalian cells are not dispersed by BFA treatment (Ward *et al*., 2001), and we surmised that ER-retained wild-type Sys1 is enriched in tER sites through interaction with PfSec24a, which is abrogated by mutation of the DxE motif. We therefore generated transgenic lines coexpressing wild-type or mutant GFP-Sys1 and PfSec24a-mRFP. Ring stage parasites were treated with BFA for 16 h and examined by live cell microscopy. The diacidic mutant, GFP-Sys1AxA, was retained in the general ER in a pattern coincident with PfSec12 (Fig. 6A), whereas wild-type GFP-Sys1DxE was found exclusively in PfSec24a-marked tER foci (Fig. 6B). As a comparison with GFP-Sys1, we examined the behaviour of a GFP fusion of the chloroquine resistance transporter PfCRT, a multispanning membrane protein that is trafficked to the digestive vacuole. In contrast to the punctate foci observed after BFA-treatment of wild-type GFP-Sys1, PfCRT-GFP was retained throughout the ER in a homogenous perinuclear pattern (Fig. S5), suggesting either that the interaction with PfSec24a is insufficiently robust to be detected under these conditions or that PfCRT does not possess an ER export motif. Similarly, a chimeric protein consisting of the first 35 residues of Exp1 fused to GFP, which would not be expected to interact with PfSec24a and is normally secreted to the parasitophorous vacuole, was also retained within the parasite in a diffuse pattern upon BFA treatment (Adisa *et al*., 2003). Although a direct interaction between diacidic ER export signals and PfSec24a awaits a more rigorous biochemical proof, our data strongly suggest that PfSec24a possesses a functional cargo binding B-site that acts to enrich selected secretory proteins at tER sites.

Discussion

The ER of *P. falciparum* processes a large number of parasite proteins destined for export to the host erythrocyte or to intraparasitic sites such as the digestive vacuole (Marti *et al*., 2005; Przyborski and Lanzer, 2005; Tonkin *et al*., 2006). Egress of these proteins from the ER uses a vesicle transport system that originates in dedicated subdomains, known as transitional ER or tER. To define the tER in *P. falciparum*, we characterized two proteins, Sec12 and Sec24, involved in the generation of ER-derived COPII vesicles. Unlike species in which Sec12 marks discrete foci, PfSec12 was found dispersed throughout the ER. Although Sec12 was initially considered a `landmark' tER protein, neither RNAi depletion in mammalian cells nor generation of a delocalized Sec12 mutant in *P. pastoris* had an appreciable effect on the integrity of tER sites (Soderholm *et al*., 2004; Bhattacharyya and Glick, 2007). Dissection of the *P. pastoris* Sec12 protein revealed that localization to defined tER sites required determinants in both the large cytosolic domain and the charged lumenal domain, likely mediating interactions with additional tER proteins and homo-oligomerization respectively (Soderholm *et al*., 2004). PfSec12 possesses a comparatively small lumenal domain that lacks

the charged regions that mediate oligomerization, suggesting that at least one determinant of tER localization is absent. Like PfSec12, GFP-tagged PfSar1 is primarily distributed throughout the ER, although some regions of enrichment were observed that may correspond to tER sites (Adisa *et al*., 2007).

To more definitively test for the presence of tER sites, we examined the parasite orthologue of the cargo adaptor protein Sec24. Unlike Sec12, the COPII coat proteins are more robust markers of tER domains, and examination of tagged PfSec24 revealed distinct foci that likely represent tER sites. Such structures in both mammalian cells and *P. pastoris* are relatively stable and can arise *de novo* or undergo fission and fusion (Bevis *et al*., 2002; Stephens, 2003). Consistent with a similarly stable population of tER sites in *P. falciparum*, we observed a relatively uniform number of tER sites in parasites of similar developmental stages, with mature trophozoites containing ~2-3 tER and a similar number of Golgi. These observations are consistent with the recent description of parasite Sec13, which is similarly localized to distinct foci that closely appose the Golgi (Struck *et al*., 2008). Late in schizogony, daughter merozoites each inherit a single PfSec24-labelled structure that likely corresponds to an apically oriented zone of vesicle budding from the nuclear envelope previously observed by electron microscopy (Ward *et al*., 1997; Taraschi *et al*., 1998; Bannister *et al*., 2000b). Segregation of the tER into daughter cells is unlikely to be a stochastic process but may involve positioning by the spindle pole body, which in polarized merozoites is located at the nuclear envelope adjacent to vesicle budding profiles (Ward *et al*., 1997; Taraschi *et al*., 1998; Bannister *et al*., 2000b). Multiplication of tER sites appears to precede both duplication of the Golgi and nuclear division. In particular, two closely situated tER sites can occasionally be seen flanking a single spot of Golgi fluorescence, suggesting that the tER in *P. falciparum* duplicates by fission. This mechanism is similar to that suggested for *T. gondii* parasites, which show co-ordinated division of tER sites and Golgi following elongation of both structures (Pelletier *et al*., 2002). However, our observations are also compatible with the *de novo* appearance of a second tER site adjacent to the first. A more precise description of these events will require time-lapse imaging in live cells to resolve these alternative scenarios.

The formation of defined sites of vesicle budding from the ER appears to correlate with increased Golgi organization. In *S. cerevisiae*, which lacks coherent tER sites and buds COPII vesicles from the entire ER, the Golgi is unstacked with individual cisterna distributed throughout the cell. In contrast, close apposition of a stacked Golgi near discrete tER sites is observed in *P. pastoris*, plant cells, and the unicellular parasites *T. gondii* and *Trypanosoma brucei* (Rossanese *et al*., 1999; Pelletier *et al*., 2002; daSilva *et al*. 2004; He *et al*., 2004). One implication of this close relationship is that the disruption of tER integrity should affect Golgi organization. This prediction was verified in *P. pastoris* using a temperature-sensitive *sec16* mutant, which disrupted discrete tER sites resulting in Golgi fragmentation at restrictive temperature. In *P. falciparum*, however, we observe discrete tER sites that abut the Golgi, yet numerous electron microscopy and early immunofluorescence studies indicate that the parasite Golgi is a rudimentary, unstacked organelle, suggesting that coherent tER sites are not sufficient to generate Golgi stacks (de Castro *et al*., 1996; Van Wye *et al*., 1996; Ward *et al*., 1997; Bannister *et al*., 2000b). The static images obtained by electron microscopy, however, may not reveal a higher order level of organization that may be highly dynamic. Indeed, recent live cell imaging of parasite GRASP and Rab6 reveals a close relationship between these early and late Golgi markers (Struck *et al*., 2008), suggesting that some degree of organization of these two compositionally distinct compartments does exist even though elaborate Golgi stacks are not observed.

The full repertoire of proteins required to make a polarized Golgi stack remains to be determined, but comparative studies of early secretory pathway organization in different organisms may hold the key to dissecting the molecular determinants of membrane

organization. From an evolutionary perspective, the absence of elaborate Golgi stacks in *P. falciparum* is likely to result from a secondary loss of this feature (Dacks *et al*., 2003). This degeneration may be explained by the functional requirements of the *Plasmodium* secretory apparatus: one of the major roles of the Golgi is the progressive modification of oligosaccharide side-chains during glycoprotein transit through the Golgi, and segregation of sequentially acting glycosyltransferases across the Golgi stack may be a mechanism to improve the fidelity and efficiency of glycoprotein processing. *P. falciparum*, however, appears to have a minimal capacity for protein glycosylation, rendering a complex Golgi structure unnecessary (Gowda and Davidson, 1999; Anantharaman *et al*., 2007).

Aside from influencing Golgi morphology, tER sites also play an important role in the selective loading of cargo proteins into transport vesicles. Enrichment of cargo proteins at tER sites is likely to be mediated by two nonexclusive determinants: acquisition of a correctly folded state (Mezzacasa and Helenius, 2002) and interaction with the COPII coat. Sec24 functions as the COPII cargo adaptor that engages secretory proteins by direct interaction (Miller *et al*., 2003; Mossessova *et al*., 2003). Of the three known cargo binding sites on yeast Sec24, the B-site is remarkably conserved across species. PfSec24a retains characteristic cysteine and arginine residues at this site that contribute to structural integrity and cargo-binding, respectively, suggesting a conserved function for PfSec24a in selective capture of cargo at parasite tER sites. In contrast, a second putative Sec24 homologue, PfSec24b, is less conserved overall and has alterations in key cargo-binding residues. Although we have yet to demonstrate that PfSec24b functions at the ER, duplication of the Sec24 component of the COPII coat is a common feature of many genomes, potentially allowing for expansion of cargo-binding duties.

Given the conservation of the PfSec24a B-site, it seems likely that signal-mediated capture into COPII vesicles facilitates ER egress in *P. falciparum*. Using a heterologous cargo protein, yeast Sys1, which possesses a diacidic ER export motif, we observed a small amount of steady-state ER accumulation of the diacidic mutant but not of the wild-type protein. This suggests a reduction in ER export in the absence of an intact sorting signal. Furthermore, accumulation of Sys1 in tER sites upon treatment with BFA was dependent on the presence of an intact export motif. Because Sys1 uses a diacidic ER export signal that interacts with the B-site on yeast Sec24, the accumulation of Sys1 in *P. falciparum* tER sites likely derives from a similar interaction with the equivalent site on PfSec24a.

Endoplasmic reticulum export signals tend to be simple protein motifs that can be difficult to analyse by traditional sequence-based search algorithms. In addition, some secreted proteins use conformation-based signals that cannot be easily predicted, further confounding the definitive identification of ER export signals by cross-species comparison (Mancias and Goldberg, 2007). Alternatively, some cargo proteins leave the ER by non-selective bulk flow, a process that is driven by stochastic sampling of the ER membrane and lumen during vesicle formation (Martinez-Menarguez *et al*., 1999; Malkus *et al*., 2002). The low efficiency of this random process may be compensated for by dramatic overexpression of proteins (Martinez-Menarguez *et al*., 1999). In *P. falciparum*, the stage-specific transcription of secretory proteins, combined with the strict temporal pace of parasite development, may suffice to drive adequate ER export of some cargo proteins in the absence of selective enrichment (Bozdech *et al*., 2003; Le Roch *et al*., 2003). Indeed, our observation that PfCRT fails to accumulate in tER sites upon BFA treatment is suggestive of stochastic ER egress of this digestive vacuole membrane protein. A detailed comparison of the mechanisms of ER export for a variety of secreted parasite proteins will be required to discriminate non-selective export from the signal-mediated capture of high priority cargo. Furthermore, the development of quantitative *in vitro* assays for COPII vesicle formation from parasite membranes will facilitate a more mechanistic understanding of the contributions of specific cargo-coat interactions to the packaging of secreted parasite proteins.

Experimental procedures

Plasmid construction

Plasmids used in this study are listed in Table 1. The GFP-SDEL and PfGRASP-GFP plasmids were kindly provided by Dr Geoff McFadden (University of Melbourne, Australia; van Dooren *et al*., 2005) and Dr Tim Gilberger (Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany; Struck *et al*., 2005) respectively. Yeast Sar1 was expressed from pTY40 (Barlowe *et al*., 1994). The bacterial expression vector pETGEXCT (Sharrocks, 1994) was used for recombinant expression of GST-PfSar1 or GST alone. The PfSar1 coding sequence was amplified by PCR from cDNA using primers p1854/p1855 (listed in the supplementary material) and ligated into the BamHI site of pETGEXCT to yield pETGEXPfSar1. Parasite transfection vectors for expressing either GFP or mRFP fusion proteins were derived from pDC-CRT-FLAG (Fidock *et al*., 2000), which was modified as described in the supplementary material.

Protein expression and PfSar1-binding assays

Recombinant GST fusions of Sar1 from *P. falciparum* (PfSar1) and *S. cerevisiae* (ySar1), or GST alone were expressed in *E. coli* BL21(DE3) codon$^+$ cells and purified as previously described (Barlowe *et al*., 1994). For GST-PfSar1 binding experiments, ~3×10^8 infected erythrocytes containing parasites expressing mRFP-PfSec12 were first stripped of the erythrocyte plasma membrane and the parasitophorous vacuole membrane by incubation with 0.05% saponin (Sigma, St Louis, MO) in PBS for 10 min, followed by centrifugation at 3200 *g*. The parasite pellet was washed with PBS and solubilized with 1 ml of 2% (w/v) *n*-octyl-β-D-glucopyranoside (Anatrace, Maumee, OH) in binding buffer (20 mM Tris, 300 mM potassium acetate, 1 mM EDTA, pH 6.8) for 30 min on ice. Insoluble material was removed by centrifugation at 13 000 *g* for 2 min. An aliquot (250 μl) of the supernatant was mixed with GST fusion proteins immobilized on glutathione agarose beads (15 μl of a 50% slurry) for 1 h at 4°C. Beads were washed three times with 0.2% n-octylβ-D-glucopyranoside in binding buffer, and resuspended in SDS gel-loading buffer. Proteins captured on beads were analysed by SDS gel electrophoresis and immunoblotting. The presence of mRFP-PfSec12 was detected using an anti-DsRed polyclonal antibody (1:15 000 dilution, BD Biosciences, Rockville, MD) and an HRP-conjugated anti-rabbit secondary antibody (1:10 000 dilution; GE Healthcare, Piscataway, NJ).

Parasite transfection and culture

Parasites (Dd2 strain) were cultured in RPMI-1640 media with 0.5% (w/v) Albumax II (Invitrogen, Carlsbad, CA) under 5% O_2, and transfected as previously described (Fidock and Wellems, 1997). Approximately 24 h post transfection, parasite cultures were treated with either 2.5 nM WR99210 (Jacobus Pharmaceuticals, Princeton, NJ) or 2 μg ml^{-1} Blasticidin S (Invitrogen), individually or in combination to select for plasmids harbouring the human *dhfr* or *bsd* selectable markers respectively. Parasite cultures were synchronized by multiple treatments with 5% (w/v) sorbitol for 15 min at 37°C.

Image acquisition

For live cell imaging, 100 ml aliquots of resuspended culture were washed once with RPMI-1640 media lacking phenol red (Invitrogen) and Albumax II, and the cells applied to poly-L-lysine coated glass-bottom culture dishes (MatTek, Ashland, MA). Adherent cells were overlaid with 1 ml of RPMI media (free of phenol red and Albumax II) containing 2 μg ml^{-1} Hoechst 33342 (Sigma) to stain the nuclei, and were imaged immediately at room temperature. In experiments involving BFA (Epicenter Biotechnologies, Madison, WI), ring-stage parasites were treated with either 5 μg ml^{-1} of BFA in 100% ethanol, or ethanol alone (final concentration

of 0.1%), for 16 h prior to imaging. BFA was included in the imaging medium where appropriate. For immunofluorescence, samples were prepared essentially as described in Tonkin *et al*. (2004), using rabbit anti-BiP antibodies (1/500 dilution, Malaria Research and Reference Reagent Resource Center, VA) and Alexa Fluor 594 goat anti-rabbit secondary antibodies (1/2500 dilution, Molecular Probes, OR). Images were acquired on either an Olympus IX81 inverted microscope (Melville, NY) with 60× N.A. 1.4 PlanApo optics and a Cooke Sensicam QE air-cooled charge-coupled device camera (Analytical Imaging Facility, Albert Einstein College of Medicine, Bronx, NY), or a Nikon TE300 inverted microscope (Melville, NY) with 100× N.A. 1.4 PlanApo optics and a Hamamatsu Orca-ERG charge-coupled device camera. Images were collected with either IPLab 4.0 (BD Biosciences) or Openlab 5.0 (Improvision, Waltham, MA) software systems, and analysed using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Acknowledgements

We thank G. McFadden and T. Gilberger for kindly providing plasmids, and members of the Fidock lab for generous help and suggestions. This work was supported by an NRSA NIH postdoctoral fellowship (F32 AI66826) to M.L., and an Investigator in Pathogenesis of Infectious Diseases Award from the Burroughs Wellcome Fund to D.F.

References

Adisa A, Albano FR, Reeder J, Foley M, Tilley L. Evidence for a role for a *Plasmodium falciparum* homologue of Sec31p in the export of proteins to the surface of malaria parasite-infected erythrocytes. J Cell Sci 2001;114:3377–3386. [PubMed: 11591825]

Adisa A, Rug M, Klonis N, Foley M, Cowman AF, Tilley L. The signal sequence of exported protein-1 directs the green fluorescent protein to the parasitophorous vacuole of transfected malaria parasites. J Biol Chem 2003;278:6532–6542. [PubMed: 12456681]

Adisa A, Frankland S, Rug M, Jackson K, Maier AG, Walsh P, et al. Re-assessing the locations of components of the classical vesicle-mediated trafficking machinery in transfected *Plasmodium falciparum*. Int J Parasitol 2007;37:1127–1141. [PubMed: 17428488]

Albano FR, Berman A, La Greca N, Hibbs AR, Wickham M, Foley M, Tilley L. A homologue of Sar1p localises to a novel trafficking pathway in malaria-infected erythrocytes. Eur J Cell Biol 1999;78:453–462. [PubMed: 10472798]

Anantharaman V, Iyer LM, Balaji S, Aravind L. Adhesion molecules and other secreted host interaction determinants in Apicomplexa: insights from comparative genomics. Int Rev Cytol 2007;262:1–74. [PubMed: 17631186]

Ayong L, Pagnotti G, Tobon AB, Chakrabarti D. Identification of *Plasmodium falciparum* family of SNAREs. Mol Biochem Parasitol 2007;152:113–122. [PubMed: 17240462]

Bannister LH, Hopkins JM, Fowler RE, Krishna S, Mitchell GH. A brief illustrated guide to the ultra-structure of *Plasmodium falciparum* asexual blood stages. Parasitol Today 2000a;16:427–433. [PubMed: 11006474]

Bannister LH, Hopkins JM, Fowler RE, Krishna S, Mitchell GH. Ultrastructure of rhoptry development in *Plasmodium falciparum* erythrocytic schizonts. Parasitology 2000b;121:273–287. [PubMed: 11085247]

Barlowe C, Schekman R. SEC12 encodes a guanine-nucleotide-exchange factor essential for transport vesicle budding from the ER. Nature 1993;365:347–349. [PubMed: 8377826]

Barlowe C, Orci L, Yeung T, Hosobuchi M, Hamamoto S, Salama N, et al. COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell 1994;77:895–907. [PubMed: 8004676]

Behnia R, Panic B, Whyte JR, Munro S. Targeting of the Arf-like GTPase Arl3p to the Golgi requires N-terminal acetylation and the membrane protein Sys1p. Nat Cell Biol 2004;6:405–413. [PubMed: 15077113]

Benting J, Mattei D, Lingelbach K. Brefeldin A inhibits transport of the glycophorin-binding protein from *Plasmodium falciparum* into the host erythrocyte. Biochem J 1994;300(Part 3):821–826. [PubMed: 8010965]

Bevis BJ, Hammond AT, Reinke CA, Glick BS. De novo formation of transitional ER sites and Golgi structures in Pichia pastoris. Nat Cell Biol 2002;4:750–756. [PubMed: 12360285]

Bhattacharyya D, Glick BS. Two mammalian Sec16 homologues have nonredundant functions in endoplasmic reticulum (ER) export and transitional ER organization. Mol Biol Cell 2007;18:839–849. [PubMed: 17192411]

Bi X, Corpina RA, Goldberg J. Structure of the Sec23/24-Sar1 pre-budding complex of the COPII vesicle coat. Nature 2002;419:271–277. [PubMed: 12239560]

Bonifacino JS, Glick BS. The mechanisms of vesicle budding and fusion. Cell 2004;116:153–166. [PubMed: 14744428]

Bozdech Z, Llinas M, Pulliam BL, Wong ED, Zhu J, DeRisi JL. The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. Plos Biol 2003;1:E5. [PubMed: 12929205]

Bretscher MS, Munro S. Cholesterol and the Golgi apparatus. Science 1993;261:1280–1281. [PubMed: 8362242]

de Castro FA, Ward GE, Jambou R, Attal G, Mayau V, Jaureguiberry G, et al. Identification of a family of Rab G-proteins in *Plasmodium falciparum* and a detailed characterisation of pfrab6. Mol Biochem Parasitol 1996;80:77–88. [PubMed: 8885224]

Chardin P, Callebaut I. The yeast Sar exchange factor Sec12, and its higher organism orthologs, fold as beta-propellers. FEBS Lett 2002;525:171–173. [PubMed: 12163183]

Crary JL, Haldar K. Brefeldin A inhibits protein secretion and parasite maturation in the ring stage of *Plasmodium falciparum*. Mol Biochem Parasitol 1992;53:185–192. [PubMed: 1501638]

Dacks JB, Davis LA, Sjogren AM, Andersson JO, Roger AJ, Doolittle WF. Evidence for Golgi bodies in proposed `Golgi-lacking' lineages. Proc Biol Sci 2003;270(Suppl 2):S168–S171. [PubMed: 14667372]

van Dooren GG, Marti M, Tonkin CJ, Stimmler LM, Cowman AF, McFadden GI. Development of the endoplasmic reticulum, mitochondrion and apicoplast during the asexual life cycle of *Plasmodium falciparum*. Mol Microbiol 2005;57:405–419. [PubMed: 15978074]

Fidock DA, Wellems TE. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. Proc Natl Acad Sci USA 1997;94:10931–10936. [PubMed: 9380737]

Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, et al. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell 2000;6:861–871. [PubMed: 11090624]

Gowda DC, Davidson EA. Protein glycosylation in the malaria parasite. Parasitol Today 1999;15:147–152. [PubMed: 10322336]

Gurkan C, Stagg SM, Lapointe P, Balch WE. The COPII cage: unifying principles of vesicle coat assembly. Nat Rev Mol Cell Biol 2006;7:727–738. [PubMed: 16990852]

He CY, Ho HH, Malsam J, Chalouni C, West CM, Ullu E, et al. Golgi duplication in *Trypanosoma brucei*. J Cell Biol 2004;165:313–321. [PubMed: 15138289]

Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O'Shea EK. Global analysis of protein localization in budding yeast. Nature 2003;425:686–691. [PubMed: 14562095]

Kirk SJ, Ward TH. COPII under the microscope. Semin Cell Dev Biol 2007;18:435–447. [PubMed: 17693103]

Le Roch KG, Zhou Y, Blair PL, Grainger M, Moch JK, Haynes JD, et al. Discovery of gene function by expression profiling of the malaria parasite life cycle. Science 2003;301:1503–1508. [PubMed: 12893887]

Lee MC, Miller EA. Molecular mechanisms of COPII vesicle formation. Semin Cell Dev Biol 2007;18:424–434. [PubMed: 17686639]

Malkus P, Jiang F, Schekman R. Concentrative sorting of secretory cargo proteins into COPII-coated vesicles. J Cell Biol 2002;159:915–921. [PubMed: 12499351]

Mancias JD, Goldberg J. The transport signal on Sec22 for packaging into COPII-coated vesicles is a conformational epitope. Mol Cell 2007;26:403–414. [PubMed: 17499046]

Marti M, Baum J, Rug M, Tilley L, Cowman AF. Signal-mediated export of proteins from the malaria parasite to the host erythrocyte. J Cell Biol 2005;171:587–592. [PubMed: 16301328]

Martinez-Menarguez JA, Geuze HJ, Slot JW, Klumperman J. Vesicular tubular clusters between the ER and Golgi mediate concentration of soluble secretory proteins by exclusion from COPI-coated vesicles. Cell 1999;98:81–90. [PubMed: 10412983]

Mezzacasa A, Helenius A. The transitional ER defines a boundary for quality control in the secretion of tsO45 VSV glycoprotein. Traffic 2002;3:833–849. [PubMed: 12383349]

Miller EA, Beilharz TH, Malkus PN, Lee MC, Hamamoto S, Orci L, Schekman R. Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. Cell 2003;114:497–509. [PubMed: 12941277]

Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. Nature 2002;415:673–679. [PubMed: 11832955]

Mossessova E, Bickford LC, Goldberg J. SNARE selectivity of the COPII coat. Cell 2003;114:483–495. [PubMed: 12941276]

Orci L, Ravazzola M, Meda P, Holcomb C, Moore HP, Hicke L, Schekman R. Mammalian Sec23p homologue is restricted to the endoplasmic reticulum transitional cytoplasm. Proc Natl Acad Sci USA 1991;88:8611–8615. [PubMed: 1924322]

Palade G. Intracellular aspects of the process of protein synthesis. Science 1975;189:867. [PubMed: 17812524]

Pelletier L, Stern CA, Pypaert M, Sheff D, Ngo HM, Roper N, et al. Golgi biogenesis in *Toxoplasma gondii*. Nature 2002;418:548–552. [PubMed: 12152082]

Przyborski JM, Lanzer M. Protein transport and trafficking in *Plasmodium falciparum-infected erythrocytes*. Parasitology 2005;130:373–388. [PubMed: 15830811]

Rossanese OW, Soderholm J, Bevis BJ, Sears IB, O'Connor J, Williamson EK, Glick BS. Golgi structure correlates with transitional endoplasmic reticulum organization in *Pichia pastoris* and *Saccharomyces cerevisiae*. J Cell Biol 1999;145:69–81. [PubMed: 10189369]

Sharrocks AD. A T7 expression vector for producing N- and C-terminal fusion proteins with glutathione S-transferase. Gene 1994;138:105–108. [PubMed: 8125285]

daSilva LL, Snapp EL, Denecke J, Lippincott-Schwartz J, Hawes C, Brandizzi F. Endoplasmic reticulum export sites and Golgi bodies behave as single mobile secretory units in plant cells. Plant Cell 2004;16:1753–1771. [PubMed: 15208385]

Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature 2005;434:214–217. [PubMed: 15759000]

Soderholm J, Bhattacharyya D, Strongin D, Markovitz V, Connerly PL, Reinke CA, Glick BS. The transitional ER localization mechanism of *Pichia pastoris* Sec12. Dev Cell 2004;6:649–659. [PubMed: 15130490]

Stephens DJ. *De novo* formation, fusion and fission of mammalian COPII-coated endoplasmic reticulum exit sites. EMBO Rep 2003;4:210–217. [PubMed: 12612614]

Struck NS, de Souza Dias S, Langer C, Marti M, Pearce JA, Cowman AF, Gilberger TW. Re-defining the Golgi complex in *Plasmodium falciparum* using the novel Golgi marker PfGRASP. J Cell Sci 2005;118:5603–5613. [PubMed: 16306223]

Struck NS, Herrmann S, Schmuck-Barkmann I, de Souza Dias S, Haase S, Cabrera AL, et al. Spatial dissection of the cis- and trans-Golgi compartments in the malaria parasite *Plasmodium falciparum*. Mol Micro-biol 2008;67:1320–1330.

Taraschi TF, Trelka D, Schneider T, Matthews I. *Plasmodium falciparum*: characterization of organelle migration during merozoite morphogenesis in asexual malaria infections. Exp Parasitol 1998;88:184–193. [PubMed: 9562421]

Tonkin CJ, van Dooren GG, Spurck TP, Struck NS, Good RT, Handman E, et al. Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. Mol Biochem Parasitol 2004;137:13–21. [PubMed: 15279947]

Tonkin CJ, Pearce JA, McFadden GI, Cowman AF. Protein targeting to destinations of the secretory pathway in the malaria parasite *Plasmodium falciparum*. Curr Opin Microbiol 2006;9:381–387. [PubMed: 16828333]

Van Wye J, Ghori N, Webster P, Mitschler RR, Elmendorf HG, Haldar K. Identification and localization of rab6, separation of rab6 from ERD2 and implications for an `unstacked' Golgi, in *Plasmodium falciparum*. Mol Biochem Parasitol 1996;83:107–120. [PubMed: 9010846]

Votsmeier C, Gallwitz D. An acidic sequence of a putative yeast Golgi membrane protein binds COPII and facilitates ER export. EMBO J 2001;20:6742–6750. [PubMed: 11726510]

Ward GE, Tilney LG, Langsley G. Rab GTPases and the unusual secretory pathway of plasmodium. Parasitol Today 1997;13:57–62. [PubMed: 15275124]

Ward TH, Polishchuk RS, Caplan S, Hirschberg K, Lippincott-Schwartz J. Maintenance of Golgi structure and function depends on the integrity of ER export. J Cell Biol 2001;155:557–570. [PubMed: 11706049]

Wickert H, Rohrbach P, Scherer SJ, Krohne G, Lanzer M. A putative Sec23 homologue of *Plasmodium falciparum* is located in Maurer's clefts. Mol Biochem Parasitol 2003;129:209–213. [PubMed: 12850265]

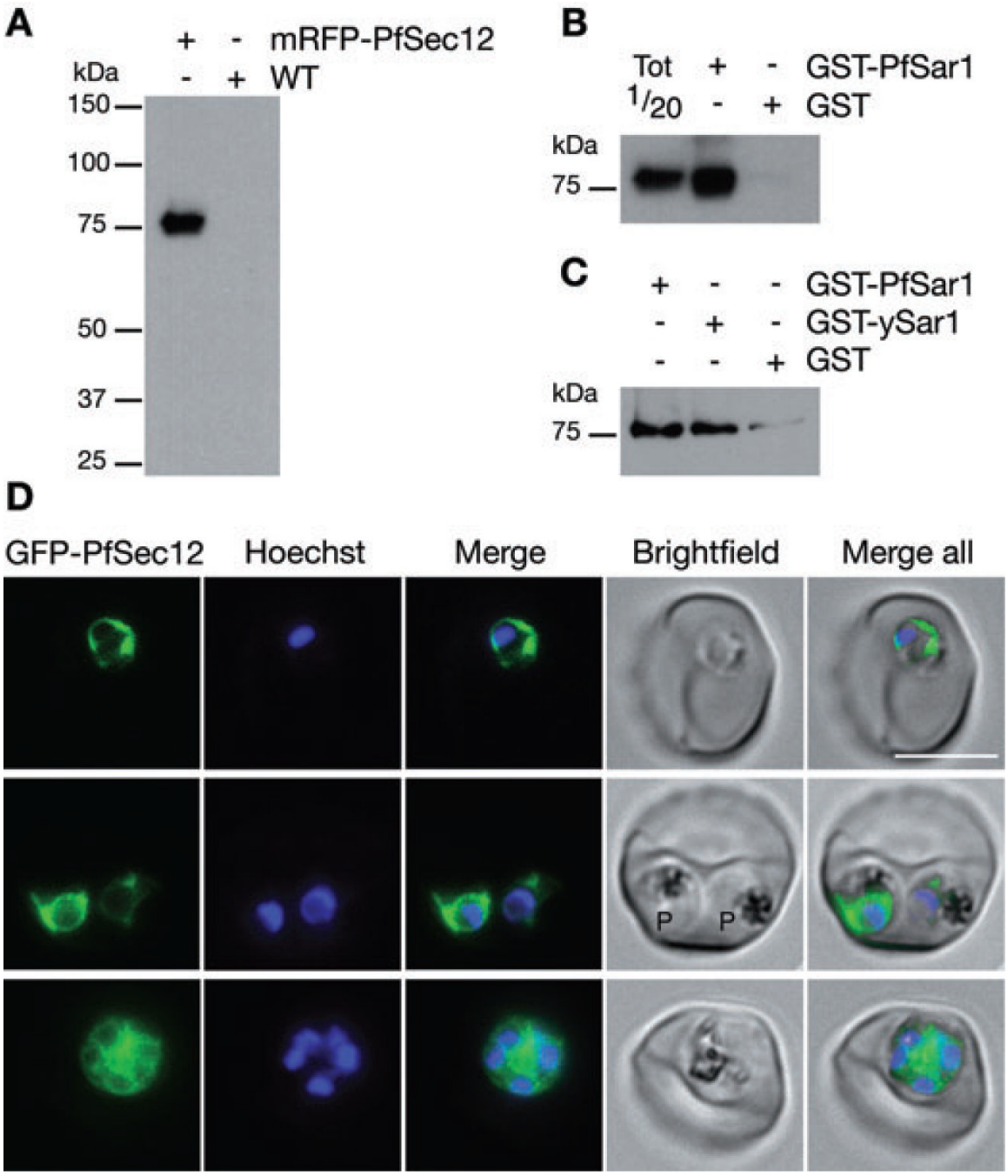

Fig. 1.

Characterization of PfSec12.

A. Immunoblot using anti-DsRed antisera detects a protein of ~75 kDa in total parasite extracts from mRFP-PfSec12 transgenic parasites but not from an untransfected wild-type (WT) control.

B. Octylglucoside-solubilized extracts from parasites expressing mRFP-PfSec12 were incubated with either GST-PfSar1 or GST alone, immobilized on glutathione-agarose beads. The amount of bound mRFP-PfSec12, as well as a fraction (1/20) of the total input material, was assessed by immunoblot using anti-DsRed antisera.

C. mRFP-PfSec12 pulldown assay using GST fusions of PfSar1, yeast Sar1 (ySar1) or GST alone, performed as in (B). mRFP-PfSec12 was efficiently captured by beads containing parasite and yeast Sar1, with a low level of non-specific binding to GST alone.
D. Live cell imaging of parasites expressing GFP-PfSec12 from the endogenous *pfsec12* 5′ UTR. Nuclei were stained with Hoechst 33342. GFP-PfSec12 is distributed in a perinuclear pattern with additional reticular protrusions characteristic of the parasite ER. Parasites at the ring, trophozoite and schizont stages are shown in the top, middle and bottom rows respectively. Note that the trophozoites (labelled P) were in a doubly infected erythrocyte. Bar = 5 μm.

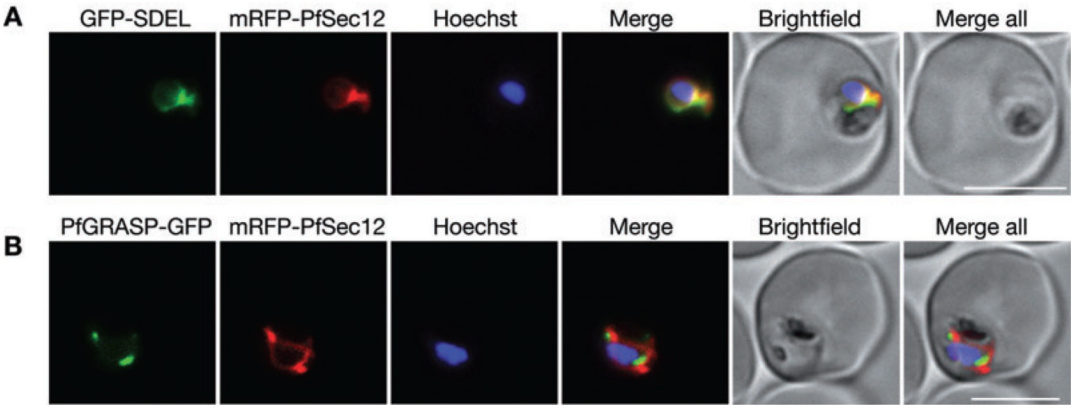

Fig. 2.

Comparison of PfSec12 localization with markers for the ER and Golgi. Parasites were co-transfected with plasmids for expression of mRFP-PfSec12 (from the native *pfsec12* 5′ UTR) with either (A) GFP-SDEL, a marker for the general ER, or (B) PfGRASP-GFP, a *cis*-Golgi marker. mRFP-PfSec12 was localized throughout the ER coincident with GFP-SDEL, in close apposition to PfGRASP-labelled Golgi. Bar = 5 μm.

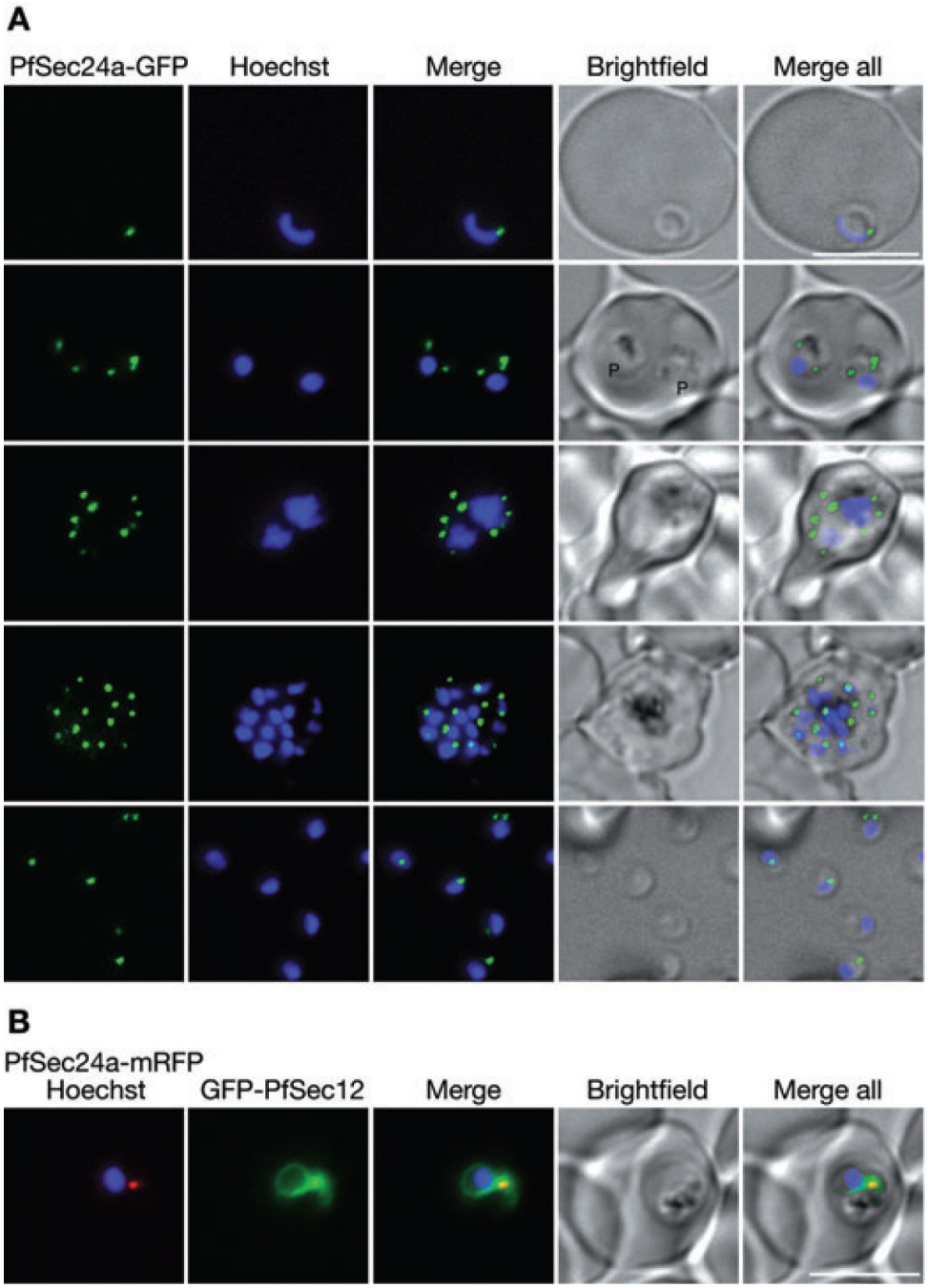

Fig. 3.

The parasite COPII protein PfSec24a labels discrete tER sites.

A. PfSec24a-GFP marks 1-2 foci in ring stages (first row) and 2-3 foci in trophozoite stages (second row, parasites labelled `P' in a doubly infected erythrocyte). Proliferation of tER sites accompanied the onset of nuclear division in early schizonts (third row). Late in the intraerythrocytic cycle, however, only a single spot of PfSec24a-GFP is associated with each nucleus in late schizonts (fourth row). Similarly, released merozoites typically possess a single spot of PfSec24-GFP fluorescence (fifth row).

B. Parasites co-transfected with PfSec24a-mRFP and GFP-PfSec12 at the trophozoite stage. PfSec24 foci appear closely associated with the nuclear envelope in mature parasites. Bar = 5 μm.

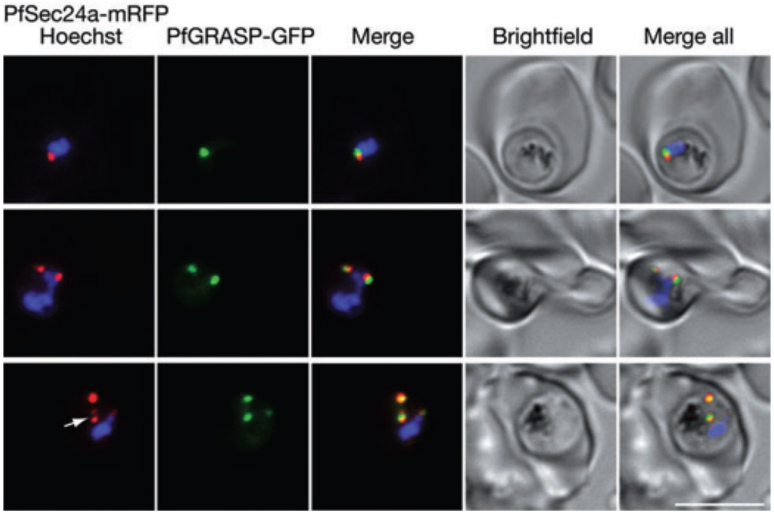

Fig. 4.

Close apposition of PfSec24a-marked tER sites and Golgi. Parasites expressing PfSec24a-mRFP and PfGRASP-GFP demonstrate a close relationship between the number and location of tER sites and Golgi, as illustrated in three representative parasites. Occasionally, two tER foci can be observed adjacent to a single Golgi spot (arrow in bottom row). Bar = 5 μm.

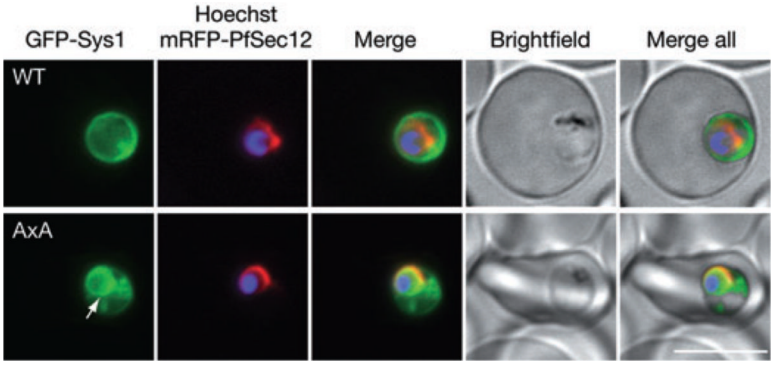

Fig. 5.

Expression of a model secreted protein, yeast Sys1. Parasites coexpressing the ER marker mRFP-PfSec12 (from the *calmodulin* 5′ UTR) and the GFP-Sys1 reporter protein with either an intact DxE export signal (WT) or a mutated AxA signal (AxA). Although both forms of GFP-Sys1 are delivered to the plasma membrane, a fraction of mutant GFP-Sys1AxA is observed in the ER (arrow). Bar = 5 μm.

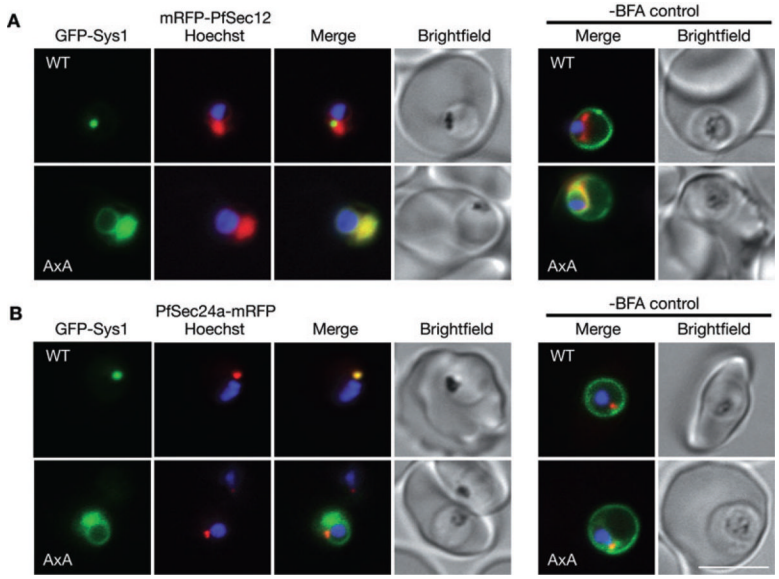

Fig. 6.

Brefeldin A (BFA) treatment causes retention of GFP-Sys1 at tER sites in an export signal-dependent manner. Parasites coexpressing either GFP-Sys1DxE (WT) or GFP-Sys1AxA (AxA) with (A) mRFP-PfSec12 (from the *calmodulin* 5′ UTR) or (B) PfSec24a-mRFP were treated with 5 μg ml^{-1} BFA for 16 h prior to imaging. A merged image of a mock-treated control is shown for comparison. ER-retained GFP-Sys1AxA colocalized with PfSec12 throughout the ER (A), whereas GFP-Sys1DxE was enriched at tER sites coincident with PfSec24a (B). Bar = 5 μm.

Table 1

Plasmids used in this study.

Plasmid	Description	Source
GFP-SDEL	ER-retained GFP-SDEL in pENTR2/3	van Dooren *et al.* (2005)
PfGRASP-GFP	PfGRASP-GFP in pARL1a$^-$	Struck *et al.* (2005)
pETGEXCT	GST-tag bacterial expression vector	Sharrocks (1994)
pTY40	GST fusion of yeast Sar1 in pGEX-2T bacterial expression vector	Barlowe *et al.* (1994)
pETGEX-PfSar1	PfSar1 in pETGEXCT bacterial expression vector	This study
pPM-*campro*-CRT-Flag-GFP	PfCRT-GFP, 1 kb calmodulin 5′ UTR (human *dhfr*)	This study
pML-*campro*-GFP(N-C)	GFP-GFP tandem, 1 kb *calmodulin* 5′ UTR (human *dhfr*)	This study
pML1	GFP-PfSec12, 1367 bp *pfsec12* 5′ UTR (human *dhfr*)	This study
pML2	mRFP-PfSec12, 1367 bp *pfsec12* 5′ UTR (*bsd*)	This study
pML3	mRFP-PfSec12, 1 kb *calmodulin* 5′ UTR (*bsd*)	This study
pML4	PfSec24a-GFP, 1230 bp *pfsyn17* 5′ UTR (human *dhfr*), Rep20	This study
pML5	PfSec24a-mRFP, 1230 bp *pfsyn17* 5′ UTR (*bsd*)	This study
pML6	yeast GFP-Sys1 (WT), 1367 bp *pfsec12* 5′ UTR (human *dhfr*)	This study
pML7	yeast GFP-Sys1 (AxA), 1367 bp *pfsec12* 5′ UTR (human *dhfr*)	This study